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The Extracytoplasmic Sigma Factor, σ^E, Is Required for Intracellular Survival of Nontypeable *Haemophilus influenzae* in J774 Macrophages

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Nontypeable *Haemophilus influenzae* (NTHi) causes a wide variety of respiratory tract infections in humans. It is capable of invading and surviving in epithelial cells and has also been shown to persist in macrophage-like cell line J774A.1. To determine the molecular mechanisms which enable NTHi to survive in an intracellular environment, differential display reverse transcriptase PCR was used to identify genes which were either induced or upregulated by NTHi residing in macrophages. Using this approach, we identified one transcript which was consistently amplified from intracellular NTHi cDNA. Nucleotide sequence analysis of this product revealed that it spanned the 3′ and 5′ ends of *rpoE* and *rseB*, respectively, which form part of the extracytoplasmic stress operon that encodes and regulates expression of alternate sigma factor sigma E (σ^E). To confirm that expression of *rpoE* was upregulated following uptake of NTHi by macrophages, an *rpoE-lacZ* transcriptional fusion was constructed, and expression of β-galactosidase activity in broth-grown NTHi was compared with expression of β-galactosidase activity in intracellular NTHi. The level of β-galactosidase activity in NTHi 4 h after phagocytosis by macrophages was found to be 100-fold higher than that of broth-grown organisms, suggesting that genes of the σ^E regulon may be important for persistence of NTHi in mammalian cells. The hypothesis that σ^E plays a role in the intracellular survival of NTHi was subsequently confirmed by the decreased ability of an *rpoE* insertion mutant to survive in macrophages.

Nontypeable *Haemophilus influenzae* (NTHi) is a nonencapsulated bacterium which forms part of the commensal flora of the human upper respiratory tract. This organism causes a variety of infections, including sinusitis, bronchitis, otitis media, and pneumonia (29). One problem associated with disease caused by NTHi is the ability of this organism to persist in the respiratory tract and cause recurrent infections once antibiotic therapy has ceased (2, 23). In the case of otitis media, recrudescent infections occurring as much as 5 months after the original onset of symptoms have been described. Such persistent reinfection is common in preschool children and can result in serious sequelae, including perforation of the eardrums and hearing impairment in adulthood (28).

Although NTHi is not generally considered an invasive microorganism, increasing evidence suggests that this bacterium is able to survive in host cells. It has been demonstrated that NTHi can penetrate and survive in Chang and human bronchial epithelial cell monolayers (22, 35). In the case of bronchial epithelial cells adhesion and invasion of NTHi are mediated by the interaction of lipooligosaccharide with the platelet-activating factor receptor (36). Intracellular organisms have also been found in biopsy samples from patients with chronic bronchitis and in NTHi-infected adenoid organ cultures, in which the organism either is clustered between adjacent epithelial cells or occurs intracellularly in mononuclear cells (11). NTHi has also been identified in macrophage-like cells in adenoid tissue obtained from children persistently infected with NTHi. These organisms were shown to be viable, suggesting that NTHi could survive macrophage-mediated killing (15). More recently, we demonstrated that some NTHi isolates are capable of surviving in mouse macrophage cell line J774A.1 for at least 72 h (6). This apparent ability of NTHi to sequester itself within macrophages and other host cells, thereby avoiding circulating antibodies and antibiotics, may contribute to its ability to cause endogenous reinfection.

The ability to survive in macrophages is a potent strategy for preventing clearance from the host and has been adopted by other intracellular pathogens, such as *Salmonella enterica* serovar Typhimurium and *Legionella pneumophila*. One feature of these organisms is that following phagocytosis they either upregulate or induce expression of a specific set of stress proteins essential for intracellular survival (13). In *S. enterica* serovar Typhimurium phagocytosis by macrophages is accompanied by de novo synthesis of more than 30 proteins (4). This adaptive response provides protection from a diverse range of antimicrobial mechanisms inherent in macrophages, including oxidative free radicals, lysosomal enzymes, and transient acidification of the phagosome. In order for NTHi to survive in macrophages, it too must be able to protect itself from the environmental stresses encountered in an intracellular environment. Identifying the genes involved in this process is therefore critical to our understanding of the host-pathogen interaction.

In this study we sought to identify genes which were either induced or upregulated by NTHi in response to the intracellular environment of macrophages. To compare the pattern of genes expressed by intracellular NTHi with the pattern of genes expressed by extracellular organisms, we used the ubiquitous *H. influenzae* uptake signal sequence (USS) as a primer in a differential display reverse transcriptase PCR (dd-RT-

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TABLE 1. Bacterial strains and plasmids used or constructed in this study

Strain or plasmid	Relevant characteristics	Source or reference
H. influenzae strains		
NT1008	NTHi clinical isolate	
Rd	Nonencapsulated variant of a serotype d strain	5
NT1008E	NT1008 σ^E::Kan	This study
E. coli DH5α		17
Plasmids		
pCRII	PCR cloning vector	Invitrogen
pBluescript SK	Ap^r cloning vector	Stratagene
pUC4K	Km^r cassette	30
pJEC62	*rpoE* promoter region in pBluescript	This study
pJEC65	*rpoE* in pBluescript	This study
pJEC67	*Xba*I fragment from pLic2A in pBluescript	This study
pJEC69	*rpoE*::*kan* in pBluescript	This study
pLK	Promoterless *lacZ* gene	
pJEC72	*rpoE'-'lacZ* in pBluescript	This study
pLic2A	*lic2* locus	19
pJEC73	*rpoE-lacZ* in *ksgA* gene in pJEC67	This study

PCR). There are approximately 1,500 USSs distributed largely at random in the genome of *H. influenzae*. Each comprises a 29-bp consensus sequence containing the core sequence 5′-AAAGTGCGGT-3′ at its 5′ end (34). When used as a primer in dd-rtPCR, this consensus sequence allows expression of a comprehensive number of genes to be monitored simultaneously. Using this approach, we determined that following phagocytosis by macrophages, the profile of genes expressed by NTHi changes, consistent with an adaptive response. We found that one of the genes upregulated during this adaptive response encodes the sigma E (σ^E) subunit of RNA polymerase (*rpoE*). In *Escherichia coli* RpoE is a prerequisite for the extracytoplasmic stress response and directs expression of a specific regulon following detection of environmental stresses or protein misfolding in the periplasm (8, 9, 24). σ^E has also recently been shown to be critically important for the virulence of *S. enterica* serovar Typhimurium. The hypothesis that σ^E plays a role in the survival of NTHi in macrophages was confirmed by the observation that the intracellular survival of an NTHi *rpoE* mutant was impaired compared to the intracellular survival of its wild-type parent (21). Genes of the σ^E regulon are therefore implicated in promotion of survival of NTHi in macrophages.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used or constructed in this study are listed in Table 1. All *H. influenzae* strains were grown in brain heart infusion (BHI) broth supplemented with hemin (10 μg ml^{-1}) and NAD (20 μg ml^{-1}). BHI agar plates were prepared by adding Bacto Agar (1.5%, wt/vol; Difco) and Levinthal's base (10%, vol/vol) (1). When required, ampicillin was used at a concentration of 50 μg ml^{-1} and kanamycin was used at a concentration of 50 μg ml^{-1} for growth of *E. coli*, and kanamycin was used at a concentration of 10 μg ml^{-1} for growth of *H. influenzae.*

DNA manipulation. Restriction and modifying enzymes were obtained from Boehringer Mannheim. Standard methods were used for restriction enzyme digestion, ligation, transformation, and preparation of plasmid DNA from *E. coli*. Transformation of *H. influenzae* was performed as described elsewhere (18). Double-stranded sequencing of cloned reverse transcriptase PCR bands was carried out as described in the Sequenase handbook (U.S. Biochemicals).

Cell lines. A phagocytic cell line, BALB/c mouse macrophage-like cell line J774A.1 (ATCC 91051511), and a nonphagocytic cell line, human epithelial cell line Hep2 (ATCC 86030501), were used. J774A.1 cells were grown in Dulbecco's modified Eagle medium (DMEM), and Hep2 cells were grown in minimal essential medium containing Earle's balanced salts solution. Both media were supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (Gibco BRL). The cells were maintained at 37°C in the presence of 5% CO_2 in a moist chamber and were subcultured every 3 to 5 days.

Invasion assays. For invasion assays (and RNA isolation), cells were seeded into six-well tissue culture plates (Costar) at a concentration of approximately 2×10^6 cells/well and incubated overnight at 37°C in the presence of 5% CO_2, and the invasion assay was carried out as previously described (6). Prior to infection, the monolayers were washed three times with phosphate-buffered saline (PBS) and then overlaid on fresh tissue culture medium without serum. Exponentially growing bacteria were then added to the monolayers at a multiplicity of infection of 100:1. Infection was allowed to proceed for 1 h, and then the monolayers were washed three times with PBS and overlaid with fresh media containing serum and 50 μg of polymyxin B sulfate per ml to kill any residual external bacteria. The efficiency of killing of extracellular NTHi by polymyxin B sulfate was determined by Craig et al. (6), who showed that polymyxin B sulfate at a concentration of 10 mg/ml can kill a suspension containing 5×10^8 CFU of NTHi per ml in 1 h. At various times after polymyxin B sulfate was added, the monolayers were again washed three times with PBS and then lysed, and serial dilutions of each lysate were plated onto BHI agar to determine cell numbers; alternatively, RNA was extracted from the intracellular bacteria. At each time a viable count analysis was also performed with the tissue culture supernatant to ensure that no viable extracellular bacteria were present.

RNA isolation from broth-grown bacteria and from intracellular bacteria. Total RNA was isolated from BHI broth-grown NTHi by the method of Figueroa et al. (12). To isolate RNA from intracellular bacteria, infected monolayers that had been incubated for 4 h in the presence of polymyxin B sulfate were washed thoroughly with PBS to remove nonviable extracellular bacteria (6). Each monolayer was then lysed with water, and the bacteria were pelleted by centrifugation at 5,000 × *g* for 10 min. RNA was then isolated immediately and stored at −80°C before it was used.

dd-RT-PCR. A random primer (5′-WWWWWYNACCGCACTTT-3′) based on the ubiquitous *H. influenzae* DNA USS was used for dd-RT-PCR (33). RNAs isolated from broth-grown and intracellular bacteria were initially subjected to reverse transcription. Each reaction mixture contained 2 μg of RNA, each deoxynucleoside triphosphate at a concentration of 20 nM, 50 ng of uptake sequence primer, 25 U of RNase inhibitor (Boehringer Mannheim), and 40 U of avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim). The reaction mixtures were incubated at 25°C for 10 min and then at 60°C for 1 h. They were then heated to 99°C for 5 min to denature the avian myeloblastosis virus reverse transcriptase. The resulting cDNA was amplified by PCR by using the uptake sequence primer. Each 50-μl PCR mixture contained 5 μl of cDNA, 100 pmol of uptake sequence primer, each deoxynucleoside triphosphate at a concentration of 200 μM, 5 μCi of [^{32}P]dCTP, and 1 U of *Taq* polymerase (Boehringer Mannheim) in buffer supplied by the manufacturer. PCR amplification was performed by using a denaturation step consisting of 95°C for 5 min before the *Taq* polymerase was added, followed by 30 cycles of 95°C for 1 min, 42°C for 1 min, and 72°C for 2 min and a final extension step consisting of 72°C for 10 min.

To visualize the PCR products, equal volumes were separately mixed with Sequenase termination solution (U.S. Biochemicals) and denatured at 90°C for 5 min before electrophoresis on a 6% sequencing gel. The gel was dried and then subjected to autoradiography with Kodak Biomax film.

Following autoradiography dd-RT-PCR bands of interest were cut from the polyacrylamide gel, and the DNA was eluted by boiling in a 0.5 M ammonium acetate–10 mM magnesium acetate–1 mM EDTA (pH 8.0)–0.1% sodium dodecyl sulfate solution for 30 min. The DNA was then ethanol precipitated and subsequently reamplified by using the conditions described above without the radioisotope. PCR products were then cloned in pCR 2.1-TOPO (Invitrogen) according to the manufacturer's instructions.

Construction of an NTHi *rpoE* mutant. The *H. influenzae rpoE* gene was isolated by PCR from an NT1008 colony template by using primers JEC11 (5′-CTTGCTCGGAAGATTCGG-3′) and JEC12 (5′-GTTAGATAAAATACTAGTGCC-3′). The amplified DNA was cloned in pCR 2.1-TOPO (Invitrogen) and then subcloned in pBluescript SK(+) by using *Eco*RI sites to create plasmid pJEC65. The *rpoE* gene fragment was mutated by inserting a kanamycin antibiotic resistance cassette isolated from plasmid pUC4K. pUC4K was digested with *Bam*HI to isolate the kanamycin resistance cassette, which was then inserted into a unique *Mfe*I site in the *rpoE* fragment in pJEC65 following Klenow treatment of all sticky ends. The resulting plasmid (pJEC69) containing the mutated *rpoE* gene fragment (*rpoE*::Km^r) was linearized with *Sac*I and used to transform NT1008, selecting for kanamycin resistance. Mutation of the chromosomal *rpoE*

gene was confirmed by PCR performed with primers JEC11 and JEC12, which revealed that in the mutant the size of the PCR product had increased by approximately 1.2 kb as a result of insertion of the Km^r cassette (data not shown).

Construction and assay of an *rpoE′-′lacZ* reporter fusion. To construct an *rpoE′-′lacZ* fusion, a PCR-amplified (using primers 5′-GGCTTTTGGATCCCC TTGCTG-3′ and 5′-GTTAGATAAAATACTAGTGCC-3′) *Spe*I-*Bam*HI fragment containing 310 bp upstream of the *rpoE* gene and 54 bp of the coding region was cloned in pBluescript SK(+) to obtain plasmid pJEC62. The 4-kb *Bam*HI fragment carrying a promoterless *lacZ* fragment from plasmid pLK was inserted into pJEC62 digested with *Bam*HI to obtain plasmid pJEC72 containing *rpoE′-′lacZ*. The fusion was inserted into the *H. influenzae* chromosome at the *lic2* locus in the nonessential *ksgA* gene (19). This was accomplished by digesting pJEC72 with *Sma*I and *Spe*I to liberate the σ^E*′-lacZ* fragment. This fragment was then inserted into pJEC67 at a unique *Hin*dIII site following end repair with the Klenow fragment. pJEC67 is pBluescript SK(+) containing a 1.7-kb *Xba*I fragment from pLic2A and has a unique *Hin*dIII site in the *ksgA* gene. The resulting plasmid (pJEC73) contained *rpoE′-′lacZ* cloned in the orientation opposite that of *ksgA* to prevent transcriptional readthrough (this was confirmed by restriction digestion). pJEC73 was linearized with *Sma*I and used to transform NT1008, selecting for Km^r colonies. Insertion of the *rpoE′-′lacZ* fusion into the *ksgA* gene was confirmed by Southern blot analysis (data not shown).

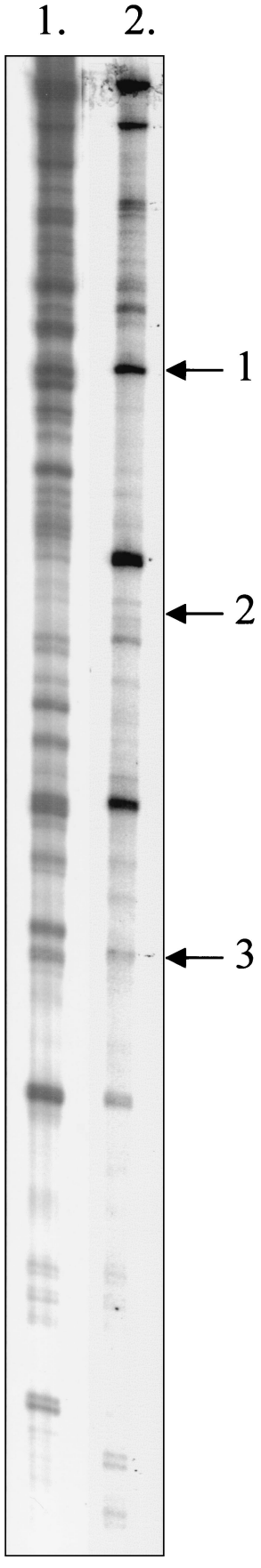
β-Galactosidase activities produced by intracellular and broth-grown bacteria were quantified with a β-galactosidase reporter gene assay kit (Boehringer Mannheim). Briefly, monolayers were infected as described above, and at zero time and 2 and 4 h after polymyxin B sulfate treatment the monolayers were washed three times with PBS and lysed, and the β-galactosidase assay was carried out according to the manufacturer's instructions. One aliquot of cells was used to determine the number of CFU of intracellular bacteria at each time, and the amount of β-galactosidase present in each sample (in femtograms per CFU) was also determined. Zero time was the time when polymyxin B sulfate was added to the infected monolayers. The number of CFU at zero time therefore represented the total cell-associated NTHi, including both adherent and intracellular organisms.

RESULTS

Comparison of the patterns of genes expressed by in vitro broth-grown and cell-associated NTHi. To establish whether NTHi elicits an adaptive response following uptake by macrophages, the pattern of genes expressed by intracellular NTHi was determined and compared to the pattern of genes expressed by broth-grown organisms. Gene expression was monitored by dd-RT-PCR by using the *H. influenzae* USS as a primer. cDNAs were synthesized from total RNAs isolated from broth-grown organisms resuspended in DMEM and organisms isolated from macrophages 4 h after phagocytosis had taken place. The cDNAs were then amplified by PCR by using the USS as a primer. The resultant PCR products were then separated by polyacrylamide gel electrophoresis. To ensure that the resultant PCR products were derived from cDNAs and were not due to contaminating genomic DNAs, control experiments were performed with purified RNAs as the templates. Using this procedure, we generated expression profiles, comprised of ladders of bands at different molecular weights, for broth-grown NTHi and intracellular NTHi (Fig. 1). A comparison of these expression profiles appeared to indicate that intracellular NTHi expressed a different repertoire of genes than organisms grown in BHI broth expressed. We identified transcripts which appeared to be either induced or upregulated in response to the intracellular environment of macrophages. Transcripts which were not expressed by NTHi or had been down regulated were also apparent. Taken as a whole, the observed differences in the expression profiles of the in vitro broth-grown organisms and the intracellular organisms suggest that NTHi does elicit an adaptive response following uptake by macrophages.

Identification of NTHi *rpoE* locus by dd-RT-PCR technique. To identify potential genes that were upregulated by NTHi following phagocytosis by macrophages, dd-RT-PCR products unique to intracellular microorganisms were excised from the polyacrylamide gel and reamplified by PCR by using the USS primer. The amplified products were then cloned into the pCR 2.1-TOPO vector, and their nucleotide sequences were determined. At this point it became apparent that many of the bands excised from the polyacrylamide gel represented a heterogeneous mixture of PCR products since the nucleotide sequences of individual clones were different. To identify the most abundant PCR product, the cloned DNAs of at least 10 clones derived from each band were T-track sequenced. The most common clone, which was presumed to represent the most abundant PCR product, was then analyzed further. Using this approach, we found that the compositions of many of the major bands in Fig. 1 were so heterogeneous that it was impossible to proceed any further with them. However, three bands (Fig. 1) were found to comprise predominantly one DNA species. The nucleotide sequence of each of these cloned DNA fragments was subsequently determined and compared to the *H. influenzae* Rd genome sequence (14) by using TBLAST. This analysis revealed that two of the cloned PCR products were fragments of the *H. influenzae* 23S rRNA gene. The third PCR product, which was 152 bp long, encompassed the 3′ and 5′ ends of the *rpoE* and *mclA* genes, which encode the σ^E subunit of RNA polymerase and a negative posttranslational regulator or anti-sigma factor, respectively (Fig. 2). Together with a third gene, *rseB*, which encodes a protein involved in negative regulation of σ^E (possibly by modulating MclA activity), *rpoE* and *mclA* form an operon which is a prerequisite for the extracytoplasmic stress response in *E. coli* (8, 25).

Transcriptional activation of *rpoE* gene following adhesion and phagocytosis by macrophages. To confirm that transcription of *rpoE* is upregulated by NTHi in the intracellualr environment of the macrophage, an *lacZ* transcriptional fusion to the promoter region of *rpoE,* was constructed. The level of *rpoE* expressed by intracellular NTHi could be quantified by using this construct. The location of the NTHi *rpoE* promoter was determined by comparison with the nucleotide sequence of the *E. coli rpoE* gene (30). Using this information, we designed a pair of primers which allowed PCR amplification of a 300-bp fragment encompassing the putative *rpoE* promoter region. To generate the *rpoE-lacZ* fusion, this fragment was then ligated to a 4-kb DNA fragment derived from PLK2 that carried a promoterless copy of the *lacZ* gene and a kanamycin resistance gene cassette. The fusion was then cloned into the *Hin*dIII site of *ksgA,* in the orientation opposite that of the open reading frame, to prevent transcriptional readthrough. *ksgA* is adjacent to *lic2A* and encodes a 16S RNA methyltransferase which confers sensitivity to the antibiotic kasugamycin. Mutations in this gene do not impair the ability of NTHi to survive in macrophages (data not shown) and provide a mechanism for introducing the *rpoE-lacZ* fusion into the genome of NTHi without disrupting the wild-type copy of *rpoE.* Accordingly, the *rpoE-lacZ* fusion was introduced into the chromosomal copy of *ksgA* by natural transformation, and transfor-



mants were selected by growth on BHI medium containing kanamycin. That the desired mutation had been introduced was confirmed by Southern hybridization analysis.

Before β-galactosidase activity in intracellular NTHi was monitored, expression of the *rpoE-lacZ* fusion was determined during growth of NT1008 in BHI broth in batch culture. This step was essential to ensure that any apparent change in the level of *rpoE* expression observed in intracellular bacteria was not solely attributable to growth-phase-dependent variation. As shown in Fig. 3A, a slight increase in *rpoE* transcription resulting in an approximately twofold increase in β-galactosidase activity was observed over a 5-h period between the mid-log phase and the stationary phase. Having established the range of β-galactosidase activity associated with broth-grown organisms, we then determined the level of *rpoE* expression induced following uptake of NTHi by macrophages. β-Galactosidase activity per CFU was measured at zero time and 2 and 4 h after phagocytosis by using Galacton Plus, a highly sensitive chemiluminescent substrate. All values recorded at these times were then compared to the β-galactosidase activity produced by bacteria resuspended in DMEM, the preinfection sample used to infect the macrophage monolayers. As shown in Fig. 3B, at zero time expression of the *rpoE* promoter had increased 10-fold compared to expression in the preinfection sample. At this time, immediately prior to addition of polymyxin B sulfate, the bacteria and macrophages had been incubated together for 1 h to allow phagocytosis to occur. The β-galactosidase activity at zero time was therefore the activity derived from all cell-associated bacteria, including both adherent and intracellular organisms. On the basis of previous studies (6), we estimated that at this stage 90% of the total population of cell-associated bacteria was bound to macrophages. By 2 h postinfection, when all extracellular, adherent organisms had been killed by the antibiotic, the β-galactosidase activity had increased dramatically to an average of 0.00027 fg/CFU, which was approximately 100-fold greater than the value obtained for the preinfection sample and 10-fold greater than the value obtained for cell-associated NTHi at zero time. This level of activity was retained at 4 h postinfection, suggesting that expression of *rpoE* had reached its maximal level in this environment (Fig. 3B). In contrast, uninfected macrophages and macrophages infected with wild-type NT1008 showed no measurable β-galactosidase activity. To confirm that the observed increases in β-galactosidase activity did not result from incubation of NTHi in DMEM, we compared expression of the *rpoE* promoter in bacteria incubated in BHI broth and expression of the *rpoE* promoter in bacteria incubated in DMEM. After 1 and 2 h of incubation in DMEM the levels of β-galactosidase activity were 2- and 1.6-fold greater, respectively, than the levels of β-galactosidase activity obtained for BHI broth-grown organisms. This verified that the observed increase in *rpoE* promoter activity was a direct result of the interaction of NTHi with macrophages.

FIG. 1. ^{32}P-labeled dd-RT-PCR products derived from BHI medium-grown *H. influenzae* (lane 1) and from intracellular bacteria recovered 4 h after infection of mouse J774A.1 macrophages (lane 2). The positions of the two 23S rRNA fragments are indicated by arrows 1 and 2. The position of the *rpoE* fragment is indicated by arrow 3.

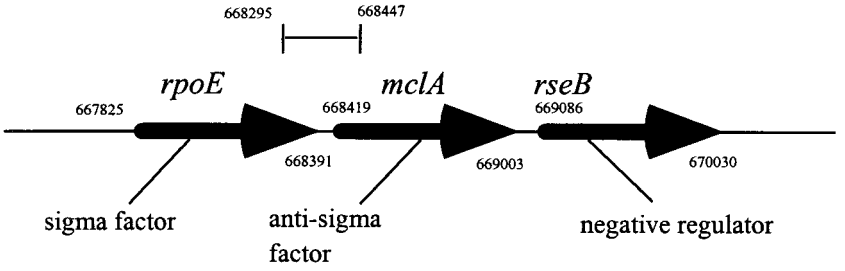


FIG. 2. Structure of the *rpoE* operon in *H. influenzae* Rd. *rpoE* encodes the alternative sigma factor σ^E; *mclA* (homologous to *rseA* of *E. coli*) encodes the anti-sigma factor predicted to reside in the inner membrane; and *rseB* encodes a potential periplasmic protein thought to interact with *mclA* in negatively regulating σ^E. The region cloned from the differential display gel and the coordinates in the genome sequence of Rd are shown.

RpoE is required for intracellular survival of NTHi. The observation that the *rpoE* gene is upregulated following phagocytosis of NTHi by macrophages suggests that σ^E may play a role in the intracellular survival of this organism. To address this question, an NT1008 *rpoE* mutant was constructed by insertional inactivation of *rpoE* by using a kanamycin resistance gene cassette as described in Materials and Methods. Loss of a functional σ^E had no apparent effect on the growth rate of NTHi, as judged by growth in BHI broth in batch culture. The doubling time of the *rpoE* mutant in BHI medium was indistinguishable from that of wild-type NTHi (data not shown). We therefore concluded that any differences in the intracellular survival rate of the mutant could not be attributed to reduced fitness caused by the *rpoE* mutation. To determine the effect of the σ^E mutation on the ability of NTHi to persist in eukaryotic cells, the ability of the *rpoE* mutant to invade and survive in murine macrophage cell line J774A.1 or human epithelial cell line Hep2 was compared to that of wild-type NTHi. As shown in Fig. 4, comparable numbers of NT1008 *rpoE*::*kan* and wild-type NTHi CFU were recovered at zero time. At this time the number of organisms recovered reflected the total number of bacteria phagocytosed, and the results suggested that the *rpoE* mutation did not impair the ability of NTHi to invade macrophages. In contrast, by 2 h postinfection the number of intracellular NT1008 *rpoE*::*kan* CFU recovered from macrophages was more than 1 log lower than the number of NT1008 CFU recovered. A comparable decrease in the number of intracellular NT1008 *rpoE*::*kan* CFU recovered from macrophages was observed at 4 h postinfection. By 24 h only 200 CFU of NT1008 *rpoE*::*kan* per well were recovered from macrophages, compared to 5×10^5 CFU of NT1008 per well. This result indicated that the σ^E mutant was deficient in the ability to survive in eukaryotic cells and provided compelling evidence that σ^E plays a role in the survival of NTHi in macrophages.

DISCUSSION

In this study we determined by using dd-RT-PCR that NTHi strain NT1008 exhibits differential gene expression following phagocytosis by macrophages. This suggests that this microorganism, like other intracellular bacteria, is able to exhibit an adaptive response to cope with the hostile environment of the macrophage. One of the genes upregulated was *rpoE*, which encodes the σ^E subunit of RNA polymerase. By constructing an *rpoE'-'lacZ* fusion we confirmed that expression of *rpoE* is enhanced in response to the intracellular environment of the macrophage. This is in agreement with the recent findings of Graham and Clark-Curtiss, who showed that *Mycobacterium tuberculosis* had elevated transcript levels of a σ^E homologue (σ^H) during human macrophage infection (16). An increase in *rpoE* promoter activity was also observed at zero time, when both adherent and intracellular bacteria were still viable. It is therefore not possible to exclude the possibility that adhesion of NTHi to macrophages may also cause upregulation of the *rpoE* promoter. However, an alternative explanation is that the observed increase in promoter activity was solely attributable to the NTHi present in the population at zero time (6) which had been phagocytosed by macrophages. On the basis of previous studies we estimated that intracellular NTHi represented approximately 10% of the total cell-associated bacteria at zero time. If this was the case, then the level of β-galactosidase activity per CFU of intracellular bacteria would be predicted to be similar to that observed for NTHi 2 h following phagocytosis.

Alternative sigma factors provide a means of regulating gene expression to maintain homeostasis when the extracellular environment changes. In *E. coli* the σ^E regulon is activated by extracytoplasmic stress resulting from the accumulation of immature outer membrane proteins or misfolded polypeptides in the periplasm (24, 25). Two-dimensional gel analysis of proteins suggests that there are at least 10 members of the σ^E regulon. These include *rpoE* itself, *htrA* (or *degP*), which encodes a periplasmic serine protease, and *fkpA*, which codes for a periplasmic peptidyl-prolyl *cis*/*trans* isomerase. Under extreme stress conditions (50°C or 10% ethanol) σ^E is also required in *E. coli* for expression of the classical heat shock sigma factor (σ^{32}) (27, 31, 32). Upregulation of σ^E in NT1008 after phagocytosis by macrophages would be expected to promote transcription of the genes of the σ^E regulon in the intracellular environment. Indeed, it has been shown with other microorganisms that this does in fact occur. In *Yersinia enterocolitica* the GsrA stress protein (an HtrA homologue) and the HtrA protein of a *Salmonella* strain are both induced by macrophage phagocytosis (10, 37). These studies were the first studies to indicate that the σ^E regulon may respond to stresses in the macrophage environment.

The activity of σ^E in *E. coli* is tightly regulated; it is known that at the transcriptional level the *rpoE* gene is positively autoregulated and at the posttranslational level it is negatively regulated by the inner membrane spanning anti-sigma factor RseA and an accessory protein, RseB. RseB has been shown to

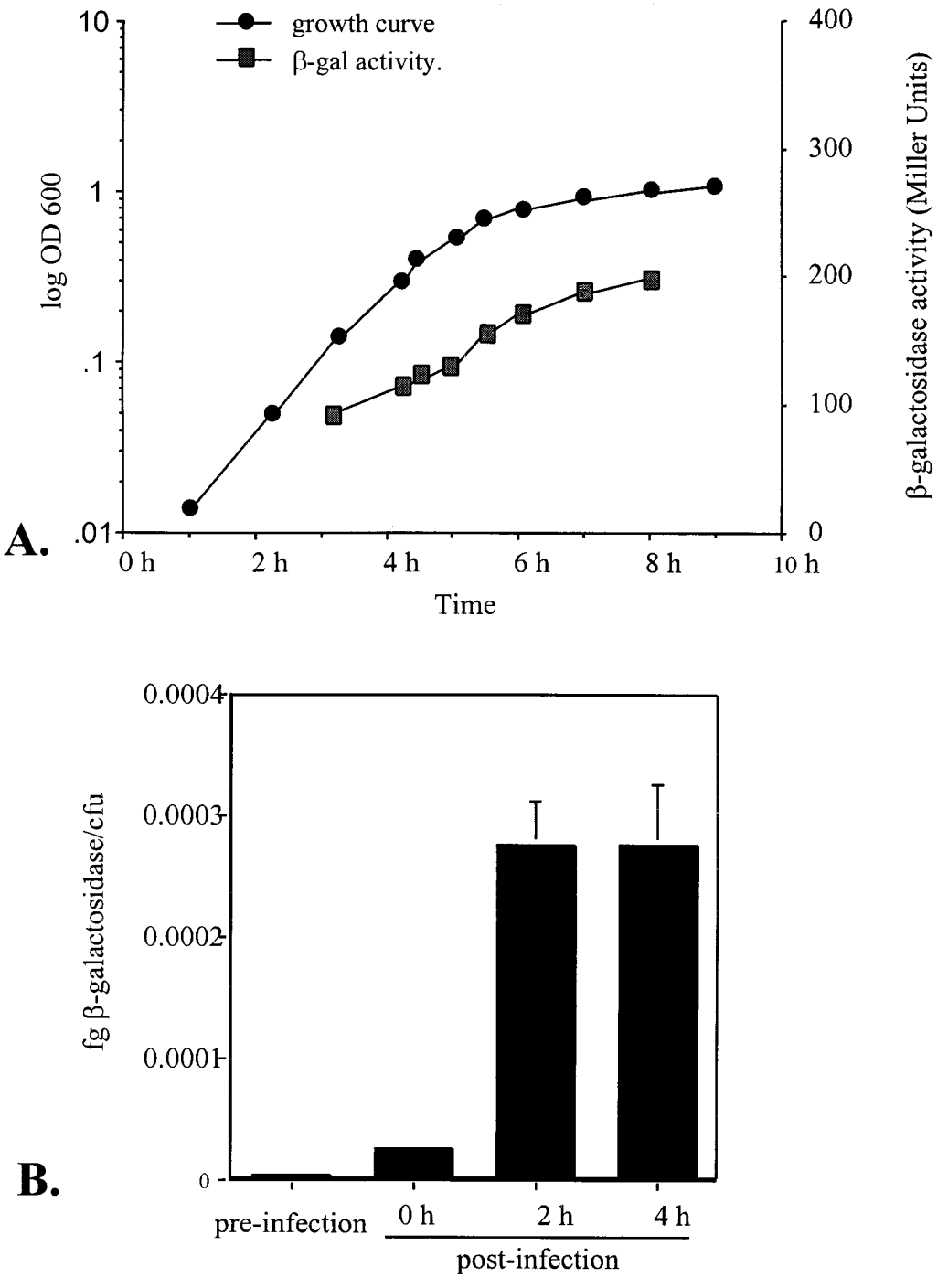


FIG. 3. (A) Regulation of σ^E expression in BHI medium. The β-galactosidase activity of NT1008 *rpoE'-'lacZ* grown at 37°C in BHI medium was determined at different times. OD 600, optical density at 600 nm. (B) Regulation of σ^E expression in the intracellular environment. The β-galactosidase activities produced by NT1008 *rpoE'-'lacZ* before infection, at zero time, and at 2 and 4 h after infection of J774A.1 cells are shown. At zero time the β-galactosidase activity was derived from total cell-associated bacteria, including both adherent and intracellular organisms. After this polymyxin B sulfate was added to kill all extracellular organisms. The error bars indicate the standard errors of the means based on the results of three independent experiments.

interact with RseA and to downregulate the σ^E pathway when it is overexpressed. *rpoE*, *rseA*, *rseB*, and a fourth gene, *rseC* (thought to encode a positive regulator of σ^E in the absence of RseB), make up an operon in *E. coli* (7, 26). Since homologues of *rseA* (*mclA*) and *rseB* are present in *H. influenzae* and are organized in the same manner that they are organized in *E. coli* (there are two *rseC* homologues, but they are at different locations), it is highly likely that NTHi possesses a similar mechanism for regulation of σ^E. In fact, homologous proteins have now been found in a number of bacterial species, indicating

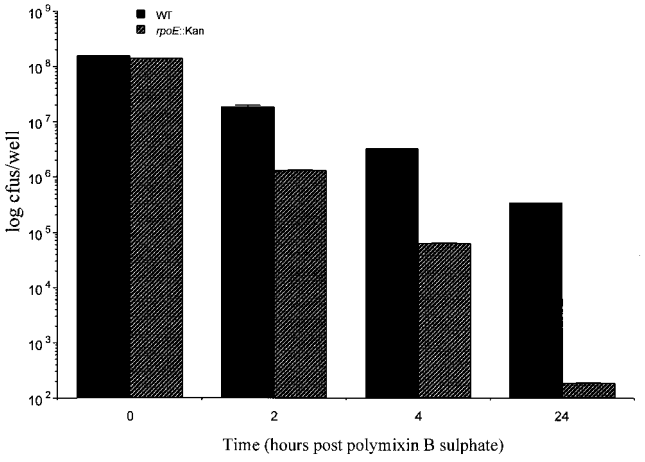


FIG. 4. Effect of an *rpoE* mutation on intracellular survival of NT1008. The ability of NT1008 *rpoE*::*kan* to survive in macrophages was examined by using mouse macrophage cell line J774A.1. The assay was performed as described in the text. The graph shows the numbers of viable bacteria inside the eukaryotic cells at zero time and at 2, 4, and 24 h after infection. Each bar represents the mean number of CFU based on the results of three independent infection experiments, and the error bars indicate the standard errors of the means. A Mann-Whitney nonparametric test was used to confirm that observed differences in the levels of intracellular survival of NT1008 and NTHi *rpoE*::*kan* were significantly different ($P < 0.05$). WT, wild type.

that there is a conserved mechanism of regulation for the σ factor and a cognate anti-sigma factor (reviewed by Missiakas and Raina [27]). However, in most cases the identity of the stimulus that leads to the release of the σ factor from the anti-sigma factor is unknown. σ^E is positively autoregulated at the transcriptional level, and in *E. coli* release of σ^E from the anti-sigma factor results in increased transcription of σ^E. However, it remains to be seen whether the elevated levels of NT1008 σ^E mRNA detected inside macrophages are due to release of σ^E from MclA and, if so, what internal factors provoke this release.

The *rpoE* mutant strain of NT1008 exhibited a decreased ability to survive inside macrophages, indicating that genes of the σ^E regulon are important in intracellular survival of this organism. It is unclear why this strain is less able to persist inside macrophages; presumably, genes of the σ^E regulon are required to cope with some of the antimicrobial mechanisms inherent in these cells. *S. enterica* serovar Typhimurium strains with mutations in the σ^E-regulated *htrA* and *fkpA* genes have been shown to exhibit decreased intracellular survival in macrophages (3, 20). The HtrA and FkpA proteins are probably required to cope with the accumulation of partially unfolded or denatured proteins in the extracytoplasmic compartments of the stressed intracellular bacteria. However, it has recently been shown that in *S. enterica* serovar Typhimurium single mutations in *htrA* and *fkpA* do not have as pronounced an effect on intracellular survival as a σ^E mutation has. This suggests that other genes in the σ^E regulon (or combinations of genes) may also be important in the survival process (20, 21). By identifying other genes of the σ^E regulon we should get a better idea of how this stress response helps NTHi survive intracellularly.

Since until recently NTHi has been considered an extracellular organism, little work has been done to look at its interaction with host cells. In this study we found, for the first time, that the σ^E locus of NTHi is clearly important for intracellular survival of this organism. Hopefully, by identifying other mRNAs that are upregulated in the macrophage environment we can start to build a picture of the mechanism(s) which this pathogen uses to persist in mammalian cells.

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